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Evaluation of a Fathead Minnow 'Pimephales promelas' Embryo-Larval Test Guideline Using Acenaphthene and Isophorone

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A set of 4 embryo-larval bioassays (2 each with isophorone and acenaphthene, respectively, were conducted with the fathead minnow, Pimephales promelas. The objective of the study was to evaluate a specific method for this type of test. The no effect levels when compared to the controls were 0.208 and 0.226 mg/l acenaphthene and 19.5 and 6.89 mg/l isophorone, respectively. The only problem encountered was in the feeding regime which may have a possibility for improvement as control weights varied.

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Toxicity testing in biological aquatic systems has been used for more than 20 years. Various kinds of tests have been conducted including static, flow-through, short-term, long-term, and static renewal tests. Species tested include most of the common species of fish and many species of invertebrates. A large bulk of the testing has been on *Daphnia magna* and the fathead minnow. There is a general consensus that much of the testing is imprecise and perhaps inaccurate. Determination of expected accuracy and precision of such testing was necessary before the use of such tests could be required by EPA.

The testing plan being reported here was to provide each of several laboratories with two chemicals and a set of guidelines, ask them to perform two tests with each chemical, and report as the primary criteria the no-effect level of each chemical on the early life-history stages of the fathead minnow. A description of this criteria is in Appendix 1. Participating laboratories were required to provide all other necessary items for following the guidelines, including water, chemical expertise, fish and testing personnel. An absolute minimum of consultation assistance was provided by the test set manager. It was decided that the guidelines must stand by themselves eventually and this was best determined by minimizing any assistance from the project chief.

Participants were asked to provide a detailed description of all the activities. Included in this detail was to be any other endpoints such as 96 or 30-day LC50s which were gathered as part of determining the no-effect level. Each participant was asked to also include a thorough discussion of interpretation and/or technique problems which were encountered during the conduct of the four required tests. The laboratory operators were also encouraged to make suggestions for rectifying any of the problems which they encountered. This report describes the participation of the Environmental Research Laboratory-Duluth in this interlaboratory comparison test.

METHODS AND MATERIALS

The water supply was sand filtered Lake Superior water; hardness 45-47 mg/l as $CaCO_3$, alkalinity 40-42 mg/l as $CaCO_3$, pH 7.8. Other chemical parameters of the water are as described in Biesinger and Christensen (1972). All water was heated in the head box by stainless steel immersion coil and aerated vigorously to bring all gases into equilibrium with the atmosphere. Water was delivered to the diluter through a rigid PVC pipe and controlled by a stainless steel and anodized aluminum solenoid valve. Experimental water temperatures were monitored continuously in three randomly assigned chambers and taken twice weekly in all chambers with a calibrated thermometer. Reported temperatures are for the calibrated thermometer readings.

The diluter (DeFoe, 1975) utilized in this work provided useful flexibility in making up the required solutions on a continuing basis. Toxic materials can be added directly by changing syringe sizes or by making different concentrations in solvent of the desired materials. This equipment was constructed of glass with silicone glue joints and teflon tubing. Teflon has been shown to be much less adsorptive than other plastics and does not contain plasticizers which may leak out and cause toxicity.

Test chambers were of glass with a minimum of silicone glass and ceramic glue. Chamber size was 46 cm x 16 cm x 18 cm containing 10 cm water with an 8 cm freeboard. Water volume was approximately 8.25 liters. Cycle time of the diluter was about 420 cycles in 24 hours and during each cycle, 0.5 liters was delivered to each chamber resulting in a 25 fold turnover in each chamber per 24 hours.

Toxic Solutions

Acenapthene was dissolved in dimethylformamide (DMF) at the required amount to add 16 ul of solvent-toxicant solution to each liter of water. At

the highest concentrations some acenapthene was noted coming out of solution at the injection point. This floating material was not sampled when the analysis for active ingredient was accomplished.

Isophorone was added directly into the diluter mixing chambers at each concentration. It is fairly water soluble and no residues were noted at any test concentrations.

Test Organisms

Embryos of the fathead minnow Pimephales promelas were removed from the cement asbestos spawning tile by gentle rolling (Gast and Brungs, 1973). A dissecting microscope was utilized to pick embryos that were undergoing cell division. Embryos were assigned in groups of five in a stratified random fashion to the screen bottomed glass embryo cup. The cups were set in petri dishes containing sufficient dilution water to keep the embryos covered during the distribution. Transfers were made by careful manipulation using an eye dropper with an enlarged opening. Embryo numbers varied from 15-35 per cup but were equal for any single test. Embryo numbers were varied by embryo availability and by choice to test the effect of this parameter.

After all embryos were transferred, the incubation cups were moved to the test chambers with minimum air exposure (less than 10 seconds) and hung on an oscillating rockerarm apparatus. This equipment cycled the embryo cups vertically causing gentle movement of the test water and maintained all test parameters in close proximity to the embryos. The fish in these tests were fed beginning the first day of hatching. This was done to prevent starvation of the earliest hatchlings. The first feeding was put directly into the embryo cups. On the fourth day of exposure all live embryos were released into the test chambers by unhooking the incubation cups from the aeration apparatus and then submerging by tipping into the chamber. Larvae were

allowed to swim out of their own volition and the cups were removed 24 hours later. This procedure minimized handling which is very stressful on newly hatched larvae. However, this procedure resulted in varying numbers of larvae in each chamber, as hatching success varied.

Feeding was accomplished by putting an aliquot of settled brine shrimp nauplii Artemia salina (Jungle brand) into each flow splitter of the diluter assuring equal distribution to each duplicate. Feeding was done at the beginning, middle, and end of an 8 hour period 5 days per week with two feedings approximately 1 hour apart 2 days on weekends. Sufficient shrimp were added so at least some were not eaten. No siphoning of tanks was done the first week after hatching, thus preventing handling injury and allowing any micro organisms in the water to grow. After 1 week approximately 5 grams per day of a very fine trout starter was added daily to each tank and tanks were siphoned every other day. Fish were killed and individually weighed to 10^{-3} grams on the 28th day post hatch. All weights were recorded and an analysis of variance and Dunnetts test were performed to determine difference from the regular and solvent control with the acenaphthene and with only the regular control in the isophorone tests.

Chemical Analysis

Chemical analyses for the toxicants were performed on all chambers initially (1st day) and finally (last day) for each test. Also twice weekly analyses were performed with duplicates alternating. All results reported were as active ingredient analyzed. Dissolved oxygen analysis was performed once a week at each concentration with duplicates alternating. Hardness, alkalinity, and pH analysis were performed twice during each test.

Analysis for acenaphthene was accomplished by using a Baird Atomic Model SFR 100 spectrofluorimeter. The fluorimeter was chosen as the instrument because it is very sensitive to the aromatic rings of the acenaphthene molecule. This structure fluoresces readily and no concentration or cleanup of the samples was needed. Water samples were taken and mixed at a ratio of 75% test solution, 25% isopropanol. This mixture was allowed to equilibrate until all of the air bubbles were gone (2-16 hours). Appropriate amounts of this solution were analyzed and the results recorded.

Standards were made by adding weighed amounts of acenaphthene to dimethyl formamide solvent and injecting appropriate aliquots into clean room temperature lake water. A standard curve was produced and used as a comparison. Spiked samples were prepared similarly from control water obtained from the experimental equipment.

Operating parameters for the fluorimeter:

Excitation Wavelength: 290 nm

Emission Wavelength: 336 nm

Excitation Slit Width: 10 nm

Emission Slit Width: 20 nm

The isophorone was analyzed by gas chromatography. Weighed amounts of isophorone were added to hexane and used for standards. The procedure for analysis was as follows: 50 ml of isophorone water solution was sampled from the test tanks and added to 50 ml redistilled hexane in 100 ml volumetric flasks. Test samples and spiked recovery samples were extracted by stirring for 1.5 hours on an electric stirrer with a teflon stirring bar. A 5 µl aliquot of the hexane layer was injected onto the GC column by automatic sampler. The mean retention time was 5.4 minutes under the following operating conditions:

Instrument: Hewlett Packard 5710A gas chromatograph with a FID detector

Column: 6 ft. x 2 mm ID glass column packed with 10% carbowax 20 M on 80/100 Gas Chrom Q

Carrier Gas: Nitrogen

Detector Temp.: 250° C

Injector Temp.: 250°C

Oven Temp.: 140° C 150 thermal.

All samples were injected twice and the mean of the two injections was reported. The precision of the instrument was periodically checked by duplicate analyses.

RESULTS

General Parameters

Temperature of the test chambers was maintained between 24.2°C and 25.6°C at all times. No excursions beyond these limits were noted in any of the four tests. Mean temperature was 25.1°C. Dissolved oxygen was always maintained at plus 90% of saturation, mainly because of the high turnover rate in the test chambers. Hardness was always between 47-48 mg/l as $CaCO_3$ and alkalinity at 38-40 mg/l as $CaCO_3$, and pH was 7.5-7.8.

The chemical analysis for acenaphthene tests 1 and 2 are found in Table 1. Data presented are analyzed concentrations in mg/l of material. There were no large excursions from the test parameters due to equipment malfunction during the acenaphthene tests.

The isophorone chemical analyses for tests 1 and 2, respectively, are presented in Table 2. All concentrations are in mg/l. Concentration 4 in test 2 had a complete failure of the toxicant addition equipment during a 16 hour period in the middle of the test which is reflected in the mean and standard deviation of test 2.

Reproducibility

The spilt sample precision was 98.4% $\pm$ 1.1% n = 8 for the acenaphthene, and 95.3% $\pm$ 6.9% n = 8 for the isophorone. Spike recoveries were 99% $\pm$ 5% for acenaphthene and 99.5% $\pm$ 7.2% for isophorone test 1 and 106.8% $\pm$ 7.1% for isophorone test 1 and 2, respectively. All reported data on the tests were adjusted for recoveries prior to running the statistical evaluations for the test data. No data were discarded.

The test concentrations all were nominally at a 0.5 factor from each other with the exception of isophorone test 1 in which the difference between the top two concentrations was only 0.33%.

Biological Results

The no-effect level for acenaphthene when compared to the solvent control was between 0.133 and 0.263 mg/l for test 1 and between 0.146 and 0.285 mg/l for test 2 (Steele and Torrie, 1960).

The no-effect levels when compared against the normal control were 0.133 to 0.263 mg/l for test 1 and between 0.593 and 1.02 mg/l for test 2. In both tests with acenapthene the solvent control fish were the largest. Also, the two lowest concentrations were larger than the normal control fish. This appears to be a usual occurrence in bioassay testing.

The no-effect levels for isophorone based on growth were between 15.6 and 22.7 mg/l for test 1 and between 4.2 and 8.8 mg/l for test 2. The total growth of the test fish was also higher in test 2 with the controls in test 1 averaging 0.141 grams and those in test 2 averaging 0.202 grams.

The effect of egg numbers was also tested as part of the work. The final low mean weight in the second acenaphthene test and the first isophorone test was at first thought to be caused by the larger number of fish used as opposed to the first acenaphthene test. To test this theory a test was conducted using 35 embryos per hatching cup (70 per concentration). The test was begun so that the larvae began to feed on Tuesday allowing 4 days of full feed before the weekend. These fish were the heaviest of the four tests.

DISCUSSION

No apparent difference in the testing procedures were found. All procedures and practices were as similar as possible. Water temperatures, flow rates, food sources, food rearing practices, feeding rates, source of fish and all similar factors were very similar if not identical. One procedure not noted in the protocol was that of feeding during the first 48 hours post hatch. In test 1 the eggs hatched on Saturday and Sunday so they were only fed twice. In test 2 they hatched mid-week and were fed three times a day during the first 2 days of life. The presence of a wider variation in size in test 2 perhaps reflects this as the standard deviation was almost double 0.026 vs. 0.017 in tests 2 and 1, respectively. This variation in growth rate was already noted by observation after about 2 weeks of testing. The guidelines called for at least one concentration to be equal to the control in growth of the test animals and at least one concentration to be significantly lower in growth, if growth was used as an end point. This requirement was met in all four of the reported tests. Variation in mean growth between tests made the use of a control absolutely necessary. One cannot compare weights of two separate tests but each test must be compared to its own control.

If solvents are used, more reproducibility is obtained when toxicnt effects are compared to solvent controls rather than to the normal controls. The acenaphthene tests are nearly identical if judged against the solvent control and about three times different when judged against the normal control. The extra growth usually found with usable solvents may make the results appear less toxic if only comparison to a normal control is made. The difference noted results from the apparent stimulation of growth by the solvent DMF which at low levels acts as a nutrient source for bacteria. This

in turn nourished microorganisms which the newly hatched fry used as food. This three-fold difference is also found in the isophorone test. Numbers of fish as noted previously are not a factor if all life sustaining parameters are maintained at a high level.

Feeding of the newly hatched fry still could be improved. If natural waters are used, certain unknown factors may interfere. The two lowest growth tests were adjacent to each other in time, but none of the usual test parameters were different in any of the tests.

The protocol as written in the guideline document appears quite easy to follow and has a limited amount of "art". This is particularly true if no sorting or thinning of the hatched fry is done. If any further work is to be done, it should be in the area of feeding during the first week post hatch because the growth differences noted took place during this time span.

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Table 1

Acenaphthene Chemical Analysis

Mean and Standard Deviation

	Control	Solvent Control	Concentration mg/l Analyzed #1	#2	#3	#4	#5
Test 1	Not Found	Not Found	0.069 ±0.009	0.133 ±0.004	0.263 ±0.061	0.474 ±0.120	1.029 ±0.200
Test 2	Not Found	Not Found	0.070 ±0.005	0.146 ±0.016	0.285 ±0.032	0.593 ±0.179	1.022 ±0.529

N = 8

Table 2

Isophorone Chemical Analysis

Mean and Standard Deviation

	Control	Concentration mg/l Analyzed #1	#2	#3	#4	#5
Test 1	Not Found	2.14 ±0.26	4.18 ±0.23	8.29 ±0.34	15.61 ±0.92	22.66 ±0.87
Test 2	Not Found	2.18 ±0.17	4.15 ±0.22	8.78 ±2.81	14.51 ±1.44	27.63 ±5.41

N = 8

Table 3

Toxicity Results

Weights of Surviving Fish and Comparison with Controls

		Control	Solvent Control	Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 5
Acenaphthene 1	$\bar{X}$	0.186	0.218	0.200	0.200	0.152[ab]	0.140[ab]	0.078[ab]
	SD	0.032	0.034	0.037	0.034	0.030	0.028	0.028
	N	31	33	40	39	50	35	31
Acenaphthene 2	$\bar{X}$	0.122	0.180	0.167	0.176	0.155[b]	0.126[b]	0.079[ab]
	SD	0.042	0.048	0.064	0.056	0.039	0.033	0.010
	N	45	50	35	49	40	52	29
Isophorone 1	$\bar{X}$	0.141	None	0.145	0.144	0.143	0.140	0.115[a]
	SD	0.047		0.066	0.060	0.052	0.040	0.031
	N	54		52	62	36	48	63
Isophorone 2	$\bar{X}$	0.202	None	0.209	0.204	0.179[a]	0.162[a]	0.171[a]
	SD	0.041		0.046	0.047	0.045	0.039	0.041
	N	68		69	63	71	71	69

[a] = 1 tail test different from normal control 0.99% level

[b] = 1 tail test different from solvent control 0.99% level

[c] = 1 tail test different from solvent control 0.95% level

Appendix 1

Guidelines for Conducting Flow-Through Early Life Stage Toxicity Tests with Fathead Minnows for Use in the USEPA, OTS-ORD Round Robin Test

1. In an Early Life Stage Toxicity Test with fathead minnows, organisms are exposed to toxicant during part of the embryonic stage, all of the larval stage and part of the juvenile stage. The organisms are examined for statistically significant reductions in survival and weight in order to determine lower and upper chronic endpoints.

   A lower chronic endpoint is the highest tested concentration (a) in an acceptable chronic test, (b) which did not cause the occurrence (which was statistically significantly different from the control at the 95% level) of any specified adverse effect, and (c) below which no tested concentration caused such an occurrence.

   An upper chronic endpoint is the lowest tested concentration (a) in an acceptable chronic test, (b) which caused the occurrence (which was statistically significantly different from the control at the 95% level) of any specified adverse effect and (c) above which all tested concentrations caused such an occurrence.

2. Not enough information is currently available concerning early life stage tests with fathead minnows to allow precise specification of details for all aspects of the test. Enough such tests have been conducted and enough aspects have been studied, however, to indicate that these Guidelines are appropriate. A prudent course of action for anyone planning to conduct such tests would be to initially conduct a

test with no toxicant to gain experience and to determine if the requirements of sections 10, 11, 19, 20, 26 and 27 can be met using the planned water, food, procedures, etc. If a solvent may be used in the preparation of a stock solution, it would also be prudent to test one or more concentrations of one or more solvents at the same time (see Section 4). General information on such things as apparatus, dilution water, toxicant, randomization of test chambers and organisms, and methods for chemical analyses, can be found in Draft #10 of the proposed ASTM Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates, and Amphibians.

3. Tests should be conducted with at least five toxicant concentrations in a geometric series and at least one control treatment. The concentration of toxicant in each treatment, except for highest concentration and the control treatment, should usually be 50 percent of that in the next higher one.

4. If a solvent other than water is used to prepare test solutions, a solvent control (at the highest solvent concentration present in any other treatment) using twice as many test organisms and test chambers as the other treatments is required in addition to the regular control, unless such a control has already been tested in the same water with the same species of fish, food, and test procedure and the water quality has not changed significantly. A concentration of solvent is acceptable only if it is (or has been) shown that concentration or a higher one does not cause an increase or decrease in survival or weight at the end of the test that is statistically significantly different from the control at the 95% level using a two-tailed test.

5. For each treatment (toxicant concentration and control) there must be at least two replicate test chambers each containing one or more embryo cups with at least 60 embryos divided equally between the embryo cups at the beginning of the test.

6. Two test chambers have been used routinely:

   a. Twenty fish have been tested in a chamber which is 16 cm x 44 cm x 18 cm high with a 16 cm x 18 cm 40-mesh stainless steel screen 6 cm from one end, with a water depth of 12.8 cm and with a flow rate of 190 ml/minute.

   b. Fifteen fish have been tested in a chamber which is 6.5 cm x 18.0 cm x 9.0 cm high with a 6.5 cm x 9.0 cm 40-mesh stainless steel screen 2.5 cm from one end, with a water depth of 4.5 cm and with a flow rate of 15 ml/minute.

   All of the above are inside dimensions. In both test chambers the water depth is controlled by a standpipe located in the smaller screened compartment with the test solution entering at the other end of the test chamber.

7. Embryo cups should be glass cylinders about 4.5 cm inside diameter and about 7 cm high with 40-mesh nylon or stainless steel screen glued to the bottom. The embryo cups must be suspended in the test chamber in such a way as to insure that the organisms are always submerged and that test solution regularly flows into and out of the cup without agitating the organisms too vigorously. A rocker arm apparatus driven by a 2 r.p.m. motor and having a vertical-travel distance of 2.5 - 4.0 cm has been successfully used, as have self-starting siphons that cause the level of solution in the test chamber to rise and fall.

8. Any water in which fathead minnows will survive, grow, and reproduce satisfactorily should be an acceptable dilution water for early life stage toxicity tests with fathead minnows.

9. A 16-hr light and 8-hr dark photoperiod should be provided. A 15- to 30-minute transition period at "lights on" and "lights off" may be desirable. Light intensities from 10 to 100 lumens at the water surface have been used successfully, but the intensity should be about the same for all test chambers. Lights should be provided by wide-spectrum (color Rendering Incex > 90) fluorescent lamps.

10. Tests should be conducted at 25° C. The temperature in each test chamber should be between 24 and 26° C at all times and must be between 20 and 28° C at all times. If the water is heated, precautions should be taken to assure that supersaturation of dissolved gases is avoided and total dissolved gases should be measured at least once during the test in the water entering the control treatment.

11. The dissolved oxygen concentration should be between 75 percent and 100 percent saturation at all times in all test chambers. At no time during the test should one test chamber have a dissolved oxygen concentration that is more than 1.1 times the dissolved oxygen concentration occurring in another chamber at the same time.

12. The flow rate of test solution through the test chambers must be great enough to maintain the dissolved oxygen concentration (see sections 11 and 22) and to insure that the toxicant concentrations are not decreased significantly due to uptake by test organisms and material on the sides and bottoms of the chambers.

13. A test begins when embryos in embryo cups are placed in test solution and ends 32 days later.

14. Embryos and fish should not be treated to cure or prevent disease or fungus before or during a test.

15. Embryos should be obtained from a fathead minnow stock culture maintained at 25° C and a dissolved oxygen concentration between 75 percent and 100 percent saturation with a 16-hr light and 8-hr dark photoperiod. Frozen adult brine shrimp has been successfully used as a food for adult fathead minnows. The maximum production of embryos by fathead minnows has been obtained in a 30 cm x 60 cm x 30 cm deep chamber with a water depth of 15 cm when 15 cm x 30 cm quadrants are formed with stainless steel screen and one male, one female and one or two substrates are placed in each quadrant. Half-round spawning substrates (Benoit and Carlson, 1977) with an inside diameter of 7.5 cm and a length of 7.5 cm have been used successfully.

16. The afternoon before a test is to begin, all of the substrates should be removed from an appropriate number of tanks in the stock culture unit and should be replaced about the time the lights are turned on the next morning. Enough (at least three) substrates with embryos on them should be removed six hours later and soaked in dilution water for two hours. For each individual substrate the embryos should be gently separated (Gast and Brungs, 1973) and removed and visually examined using a dissecting scope or a magnifying viewer. Empty shells and undeveloped and opaque embryos should be discarded. If less than 50 percent of the embryos from a substrate appear to be healthy and fertile, all the

embryos from that substrate should be discarded. Single embryos with no fungus or partial shells attached are preferable, although embryos with partial shells attached and clumps of two or three embryos (with or without separation) have been used successfully. An approximately equal number of acceptable embryos from one substrate should be impartially distributed to each embryo cup and the process repeated for at least two more substrates until the proper number of embryos have been placed in each cup to give at least 60 embryos per treatment. The embryo cups should be standing in dilution water when the embryos are being distributed and then the cups should be randomly placed in the test chambers.

17. Twenty to 24 hours after they are placed in the embryo cups, the embryos should be visually examined under a dissecting scope or magnifying viewer and all dead embryos should be counted and discarded. Embryos that are alive but heavily fungused should also be counted and discarded. Forty to 48 hours after the start of the exposure all dead and heavily fungused embryos should be counted and removed. The remaining healthy, fertile embryos should be impartially reduced to the desired number of test organisms (at least 30 per treatment). If more than about 35 percent of embryos in the control treatment are discarded within the first 48 hours of the test because they are dead or heavily fungused, it will probably be cost-effective to restart the test. In addition, if toxicant related effects are seen at 48 hours, it will probably be cost effective to restart the test since all of the toxicant concentrations will probably cause adverse effects. Each day thereafter dead embryos should be counted and discarded.

18. In each treatment, when hatching is about 90 percent complete or 48 hr after first hatch in that treatment, the live young fish should be counted and the live fish that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic or grossly abnormal in either swimming behavior or physical appearance should be counted. All of the normal and abnormal live fish should be released into the test chambers. Unhatched embryos should be left in the cups and released into the test chamber when they hatch. The range of time-to-hatch (to the nearest day) in each cup should be recorded.

19. A test should be terminated if the average percent of embryos (based on the number of embryos after thinning) that produce live fry for release into test chambers in any control treatment is less than 50 percent or if the percent hatch in any control embryo cup is more than 1.6 times that in another control embryo cup.

20. The flow rate, size of the test chamber and the amount of food added should be such that the average weight of the control fish at the end of the test would not be significantly greater if only half as many fish were tested per test chamber.

21. Each test chamber containing live fish over two days old must be fed live newly hatched brine shrimp at least two times a day at least six hrs apart (or three times a day about four hours apart) on days 2-5 after hatch and at least five days a week thereafter. They must be fed at least once a day on all other days. Other food may also be provided in addition to the above. The amount of food provided to each chamber may be proportional to the number and size of fish in the chamber, but each chamber must be treated in a comparable manner. Quantifying the

amount of live newly hatched brine shrimp to be fed is difficult, but the fish should not be excessively overfed or underfed. A large buildup of food on the bottom of the chambaer is a sign of excessive overfeeding. A sign of not feeding enough of the right kind of food is that in a sideview the abdomen does not protrude.

22. Test chambers should be cleaned often enough to maintain the dissolved oxygen concentration (see sections 11 and 12) and to insure that the toxicant concentrations are not decreased significantly due to sorption by matter on the bottom and sides. In most tests if the organisms are not overfed too much and the flow rate is not too low, removing debris from the bottom once or twice a week should be adequate. With some toxicants that promote growth of bacteria the sides and bottoms should be cleaned more often. Debris can be removed with a large pipette and rubber bulb or by siphoning into a white bucket. A dark tip on the pipette or siphon should help fish avoid being sucked up, but the pipette or bucket should be examined to insure that no live fish is discarded.

23. Temperatures should be recorded in all test chambers once at the beginning of the test and once near the middle of the test. In addition, temperature should be recorded at least hourly in one test chamber throughout the test. The dissolved oxygen concentration should be measured in each treatment at least once a week during the test. Hardness, pH, alkalinity, and acidity should be measured once a week in the control treatment and once in the highest toxicant concentration. The concentration of toxicant should be measured at least twice a week in each treatment.

24. Dead fish should be removed and recorded when observed. At a minimum 11, 18, 25 and 32 days after the beginning of the test, the live fish should be counted and the fish that are visibly (without the use of a dissecting scope or magnifying viewer) lethargic and grossly abnormal in either swimming behavior or physical appearance should be counted.

25. The fish should not be fed for the last 24 hours prior to termination on day 32. At termination the weight (wet, blotted dry) of each fish that was alive at the end of the test should be determined. If the fish exposed to toxicant appear to be edematous compared to control fish, determination of dry, rather than wet, weight is desirable.

26. A test is not acceptable if the average survival of the controls at the end of the test is less than 80 percent or if survival in any control chamber is less than 70 percent.

27. A test is not acceptable if the relative standard deviation (RSD = 100 times the standard deviation divided by the mean) of the weights of the fish that were alive at the end of the test in any control test chamber is greater than 40 percent.

28. Data to be statistically analyzed are:

    (A) percent of healthy, fertile embryos at 40-48 hours

    (B) percent of embryos that produce live fry for release into test chambers

    (C) percent of embryos that produce live, normal fry for release into test chambers

    (D) percent of embryos that produce life fish at end of test

    (E) percent of embryos that produce live, normal fish at end of test

(F) weights of individual fish that were alive at end of test.

Although item A is based on the number of embryos initially placed in embryo cups, items B, C, D, and E are based on the number of embryos after thinning.

29. Dichotomous data (live-dead, normal-abnormal) should be analyzed using contingency tables (Sokal and Rohlf, Biometry, 1969, p. 587) or log linear techniques. For weight data the individual fish are used as the replicates unless a two-tailed F test indicates that differences between replicate test chambers are not negligible. Weight data may be analyzed using (Steel and Torrie, Principles and Procedures of Statistics, 1960, p. 111) should be used to identify treatments producing weights that are statistically significantly lower than those of the controls at the 95 percent level.

30. Although the results of the analyses of all six types of data in section 28 should be reported, the lower and upper chronic endpoints are only based on statistically significant reductions in survival and weight at the end of the test (items D and F). Item A is apparently relatively insensitive and item B is included in item D. In addition, abnormal fish seem to weigh less than normal fish and so will be covered in item F. Also, since the distinction between normal and abnormal is subjective, this kind of data is expected to be less reproducible from one investigator to another than the other kinds of data. Although items D and F are considered primary, the other items are included because they may provide useful information.

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